THESIS

THROMBOPLASTIC ACTIVITY OF THE ATHEROSCLEROTIC ARTERIAL WALL

C. CH. TER HAAR ROMENY-WACHTER

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. G. SEVENSTER, HOOGLERAAR IN DE FACULTEIT DER GODGELEERDHEID, PUBLIEK TE VERDEDIGEN OP 24 JANUARI 1962 TE 16 UUR

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PROMOTOR: PROFESSOR DR. M. VINK

Aan hen die mijn medische studie
mogelijk hebben gemaakt

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On matters concerning the determination of tissue thromboplastic activity advice was given by Dr. E. A. Loeliger, of the Coagulation Laboratory of the Department of Internal Medicine, University Hospital, Leiden, who also provided working facilities.
Various departments of the University Hospital, Leiden, gave access to the material on which these experiments are based: from the Department of Pathological Anatomy were obtained aortas of young individuals; from the Department of Surgery the sections of atherosclerotic artery; from the Department of Thoracic Surgery the sections of the aorta which had been removed because of coarctation. Dr. J. Zeldenrust (State Laboratory for Forensic Medicine, Den Haag) and Dr. H. E. Schornagel and Dr. C. B. F. Daamen (Central Pathological-Anatomical Laboratory, Rotterdam) also kindly provided several aortas of young individuals. C. M. van Gent, chem. drs., as a member of the atherosclerosis-thrombosis research team in charge of the phospholipid group, developed the methodology for the separation of the phospholipids and provided valuable guidance in chemical matters.
Thanks to the co-operation of Dr. E. F. Drion (Statistics Department of T.N.O.), a method was evolved for expressing results of the thromboplastin generation tests. The technical assistance of Mrs. G. H. Rosman-Pettersson and subsequently Miss C. C. H. van Buuren is gratefully acknowledged; their loyal help has greatly facilitated the task in hand.

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INTRODUCTION

But if facts without ideas are blind,
ideas without facts are sterile

BIGGS AND MACFARLANE,
Human Blood Coagulation and its Disorders.

Each cell, every organ, every limb of the human body is entirely dependent on its arterial blood flow. Without circulation death of the organism is unavoidable: sudden death when the arterial blood flow is acutely stopped, a slow dying when the arterial blood flow is not completely interrupted, but diminished to a degree where it is insufficient. The reaction of the human body to any restriction of the bloodstream is to recruit help from other arterial structures and it will eventually train capillaries and arteriolae to take different routes and proportions in an attempt to make up for the lost circulation.

Statistics show, however, that the body is not always able to accommodate itself sufficiently to the interference in the circulation. In most civilised countries vascular (arterial) disease is already at the top of mortality lists or is heading for it. Not only is the deathrate from this cause becoming higher (which could be accounted for by a longer life-span), but the disease also strikes at a much younger age than it used to.

What is the nature of the accrescence on the wall which narrows the lumen and hampers the bloodstream? Two maladies present themselves for discussion: atherosclerosis and (arterial) thrombosis. To point to either one or the other as the sole evil seems ill-advised, for morphological studies reveal that most fatal occlusions of arteries show signs of a thrombotic process, while the arterial wall at the site of an obstruction can never be said to be free from the stamp of atherosclerosis.

With these observations in mind it seems feasible to conclude that both atherosclerosis and thrombosis play a part in the occlusion of arteries. About the precise relationship of the two processes few specific data exist.

This lack of facts has procured a host of hypotheses, some of which have hardly left their mark, while others still persist after the lapse of

a century. Virchow, for instance, was the first of a long series of investigators to postulate that the atherosclerotic lesion comes into being through the precipitation of substances from the bloodstream. Virchow was fully aware of the occurrence of thrombi but ranged them under depositions of a secondary nature. He came to the conclusion that the thrombus becomes superimposed on the atherosclerotic lesion. The theory of Rokitansky originated at about the same time; he believed thrombosis and atherosclerosis to be much more intricately linked. According to this hypothesis — for the revival of which all credit goes to Duguid — an apposition of fibrin at the arterial wall is the initial occurrence. Endothelium then covers up this minute 'thrombus', as it is wont to do with any corpus alienum. A repetition of the process eventually leads to a thickening of the arterial wall up to a point where the metabolism of the wall begins to suffer, resulting in degeneration and necrosis, secondary deposition of lipoid material and still more additions of a thrombotic nature.

Medicine — because of its obligations — cannot always afford to wait for hypotheses to be proved or disproved. Consequently reports are appearing of the long-term use of anticoagulants in occlusive arterial disease in order to forestall the occurrence of thrombosis in definitely atherosclerotic patients; and at the same time accounts are given of trials with rigorous diets, low in certain lipids, high in others, the presumed purpose of which is to prevent — via a manipulation of the lipid levels of the blood — the deposition of lipids in the arterial wall.

The thousands of autopsy surveys carried out since the classical work referred to have led in the course of the years to the conclusion that no individual can expect to evade atherosclerosis. At the same time there exists no correlation between the degree of atherosclerosis of the arteries and the clinical manifestations thereof. Furthermore it has been reported that populations with a low death rate from occlusive arterial disease nevertheless show atherosclerotic arteries. These observations reveal that atherosclerosis is a universal affliction which need not necessarily lead to any symptoms, let alone disaster. One might, then, cautiously infer that it may be the concurrence of thrombosis with atherosclerosis which decides the fatality of the process.

In order to test this hypothesis it is imperative to collect facts concerning the incidence of thrombosis in the atherosclerotic artery.

An objective approach to the subject is bedevilled by the generally existing conception that in the formation of arterial thrombosis atherosclerosis constitutes the predisposing or even the conditional factor. This conception, which tends to become accepted as a fact, is based on the following considerations. Firstly the atherosclerotic lesions encroach upon the lumen which results in changes of haemodynamics, the simplest of which is a slowing down of the bloodstream. It has been assumed that such alterations have an important influence on thrombogenesis.

Secondly there is the matter of contact: while blood will remain fluid for hours, when contained in a clamped (intact) vein or in a paraffin-coated glass tube, even the slightest roughness of the vessel lining suffices to bring about the rapid formation of a coagulum. It is conceivable then that the atherosclerotic wall, especially in a stage where the endothelial lining becomes defective, offers just such a rough surface to the bloodstream as to provoke the formation of a thrombus. Not until the initial phase of the blood coagulation mechanism is extensively and precisely known will the importance of the phenomenon of contact activation in relation to the atherosclerotic process be gauged.

A third consideration is that development of the atherosclerotic lesion elicits certain reactions of the tissues, and is associated with the accumulation of certain substances, which might have a trigger-effect on the coagulation mechanism.

It is with the last aspect of the relationship between atherosclerosis and thrombosis that this dissertation is concerned.

The object of the present investigation is:

- to examine the tissue thromboplastic potentiality of homogenates of the arterial wall;
- to investigate the thromboplastic activity of certain substances extracted from the atherosclerotic wall which might possibly bear a relationship to occlusive vascular disease, namely the phospholipids; and
- to compare the above findings — pertaining to the diseased arterial wall — with data obtained from the non-diseased arterial wall.

Atherosclerosis is a variable combination of changes of the intima of arteries (as distinguished from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes (Wld Hlth Org. techn. Rep. Ser., 1958).

Coagulation factors:

I – fibrinogen
II – prothrombin
IV – calcium
V – proaccelerin
VII – proconvertin
VIII, IX – antihaemophilic factors
X – Stuart-Prower factor
XI – plasma thromboplastin antecedent (P.T.A.)
XII – Hageman factor

Foreign surface is a surface other than the endothelial lining of normal human blood vessels.

Thromboplastic activity is a general non-specific designation for any substance which shortens the recalcification time of normal plasma (Biggs and MacFarlane 1957).

The Latin '*coagulum*', the Greek 'θρομβος', and the contemporary '*clot*' are essentially the same designation for the phenomenon resulting from solidification of ingredients from the blood, the proportion of which may vary, while fibrin constitutes the indispensable element which renders the phenomenon recognizable.
In order to avoid confusion we have reserved the word 'clot' for the process of fibrin formation in vitro. 'Thrombus' is used in the Continental sense of 'deposition thrombus', meaning a coagulation process at the vascular wall in vivo; 'coagulum' refers to an intravascular generation of fibrin, such as may occur during life and post mortem, and the origin of which need not necessarily have any connexion with the vessel lining.

CHAPTER I

BIBLIOGRAPHIC REVIEW

§ I.I LIPIDS AND OCCLUSIVE VASCULAR DISEASE

In current publications the relationship between occlusive vascular disease and lipids is very much in evidence, so much in fact, that almost anybody attempting to orient himself will at some moment be assailed by a certain 'dégout' for fats. This should be no occasion for discouragement, however, for perseverance is ultimately recompensed by the interesting vista acquired of the interplay of almost all of the lipid component in the intricate pattern of the aetiology and pathogenesis of atherosclerosis.

The typical cholesterol-like crystals in atherosclerotic lesions were described as early as the first half of the nineteenth century and in 1856 Virchow (b) wrote '. . . diese rauhen Stellen geben zu Niederschlägen aus dem Blute Anlass, die aber eben so wenig wesentlich für die fettige Usur sind, wie es die Cholestearinbildung für den atheromatösen Prozess ist; beide haben die Bedeutung späterer secundärer Veränderungen'. Virchow's contemporary Rokitansky regarded as the primary constituent among the deposits the 'Ausscheidung eines Proteinkörpers aus dem Blute' (fibrin) and in his opinion too cholesterol was of secondary importance. In 1920 Wells still regards the rôle of cholesterol as not essential in concluding that 'the large flat plates of cholesterol may be found in any tissue in which cells are undergoing slow destruction and where absorption is poor'.

A reversal of the rôle of cholesterol from the static to the dynamic originated to a great extent in the fortuitous discovery of Ignatowski (1908) that the aortas of rabbits fed large quantities of meat developed fatty accumulations resembling atheromatous lesions in man, the instrumental element in the diet being identified as cholesterol by Anitschkow in 1913, the presence of which lipoid substance had already been demonstrated in the human aorta by Windaus (1910). The observation of Anitschkow, coupled with that of the prevalence of fatty changes noticed in the arteries of diabetics and hypercholesterolaemics opened many people's eyes to the possibility of a dietary

factor in the development of atheromatosis, and has led to a continuous flow of experimental work ever since.

Concerning animal experiments there is still much controversy about the similarities and dissimilarities between the experimentally produced lesions and atherosclerotic lesions in man (e.g. Pollak 1951; Duff and McMillan 1951; McLetchie 1952; Rannie and Duguid 1953); Groen and van der Heide 1959). Even the apparent success reported of a procedure by which coronary obstructions are simulated in rats (Wilgram 1959; Thomas and Hartroft 1960) must be viewed with scepticism as long as histological examination reveals the occlusions not to be related to the specific vascular lesions. Another source of dissatisfaction is the enormous discrepancy between experimental and physiological conditions.

Epidemiological studies show that some populations are relatively free from death due to occlusive vascular disease while others are not. This has also been interpreted as pointing to a dietary factor in the pathogenesis of atherosclerosis (e.g. Snapper 1941; Keys at al. 1959). Faulty syllogistic reasoning has led many into thinking that a population with a low deathrate from cardiovascular accidents actually does not suffer from atherosclerosis of the arteries. It has been reported that this is positively not the case (Laurie and Woods 1958; Strong et al. 1959). In evaluating epidemiological reports it must be borne in mind that the deathrate as represented by occlusive vascular disease is not a standard for the occurrence of atherosclerosis, but a standard for the process causing occlusion, to which atherosclerosis may be a contributing factor. This point, stressed before by others (Morris 1951; Harrison 1960; Crawford 1961), cannot be over-emphasized, considering its repercussions on the interpretation of results in this field of research.

It is chiefly to the assumed relationship between diet and the accretions on the arterial wall that the great abundance of hypotheses concerning the various lipid components of the blood can be attributed (e.g. Swank and Wilmot 1951; Ahrens 1957; Keys and Grande 1957 (c); Dock 1958; Patil 1960). By comparing the level of a given lipid in the blood of a population susceptible to the disease with that in a non-susceptible one, or by comparing within a single population a group of patients suffering from occlusive vascular disease with a group of apparently normal controls, indications are sought for that lipid being of elementary importance in atherogenesis. Morrison et al. (1948) determined the serum cholesterol (Morris 1960) of

200 patients with acute coronary occlusion within 48 hours after admission to the hospital; they found hypercholesterolaemia in 68 % of the 75 patients who were under, and in 52 % of the 125 patients who were over 60 years of age. Gertler, Woodbury, Gottsch, White and Rusk (1959) found that the cholesterol content of the serum correlated better with the occurence of coronary-artery disease than, for instance, the serum phospholipid content or anthropometric indices. Page and Lewis (1959) followed up 1107 normal men for seven years; several developed ischaemic heart disease without any forewarning in the form of alterations of cholesterol or lipoprotein content of the blood. These results are only a few examples of many which do not contribute towards the impression that there exists unequivocally a vital connexion between serum cholesterol level and occlusive vascular disease. Although other studies have established that a weak statistical correlation does exist, the tenuous nature of this connexion reduces the value of reports of trials with various diets, where not seldom the lowering of serum cholesterol is assumed to be beneficial.

Gertler et al. (1950) confirmed the ratio between the concentrations of total cholesterol and of phospholipids (C/P ratio) as an atherogenic index and it has been examined as such by numerous authors (e.g. Adams 1959). The development by Gofman of the ultracentrifugal technique for the separation of lipoproteins (see, for example, Gofman and Jones 1950) was followed by a concentration of attention on a possible relationship between certain groups of lipoproteins and atherosclerosis.

Snapper (1941) made mention of the unsaturated fatty acids and Burr (1944, as cited by Keys, 1956) of the poly-unsaturated 'essential' fatty acids with regard to vascular disease; Alfin-Slater et al. (1954) suggested that the esters of cholesterol have a function in the transport and metabolism of fatty acids. It was Sinclair (1956) who linked the hypotheses, and maintained that the modern diet lacks essential fatty acids (E.F.A.) and is as such responsible for the increase in cardiovascular deaths in the western world. Sinclair postulated that cholesterol for want of E.F.A., is forced to a misalliance with saturated fatty acids, the resulting abnormal esters being readily deposited in the body, for instance in the arterial wall.

This not unattractive hypothesis has been tested by various authors. James and Lovelock (1958) were unable to demonstrate a lower quantum of E.F.A. in the serum cholesterol esters of coronary patients

and of hypercholesterolaemic subjects as compared with that of controls. Böttcher et al. (1960 (a)) reported that the percentage of poly-unsaturated acids in the aorta cholesterol esters is higher, and the percentage of saturated acids is lower, in severely diseased vessels than in relatively non-diseased ones. Mead and Gouze (1961), who also examined intima-plus-media preparations, found that the cholesterol ester fatty acids (C.E.F.A.) were virtually identical at all stages of atherosclerosis. On the other hand Swell et al. (1960) detected marked differences between the C.E.F.A. of far-advanced plaques, in which oleic acid and saturated fatty acids dominate, and those of the media, where linoleic acid constitutes the major part. Nelson et al. (1961) made the fatty spots the object of their investigation and concluded that the C.E.F.A. of both intima and media at the site of the lesion were richer in oleic acid than those of the adjacent, unaffected tissue. In contrast to the investigations of James and Lovelock, and Böttcher et al., those of Nelson et al. and of Swell et al. do offer some support for Sinclair's hypothesis.

The authors mentioned above were collecting information concerning a possible relationship between a deficiency of E.F.A. and occlusive vascular disease, mediated through the formation of 'abnormal' cholesterol esters. The school of Keys et al. (1957 (b)) and of Bronte-Stewart et al. (1956) have directed their investigations towards the impact of E.F.A. on the cholesterol content of the blood, a quite different matter. According to Bronte-Stewart a diet rich in unsaturated fatty acids lowers the serum-cholesterol level in non-ambulant patients, whereas Keys states that the serum cholesterol is unaffected by alterations in the E.F.A. content of the diet, but wholly determined by the total amount of fat-calories of the diet. It is now beginning to emerge that both factors may have a more or less independent effect on serum cholesterol.

After cholesterol, lipoproteins, cholesterol esters, and fatty acids, the triglycerides have attracted attention: Albrink and Man (1959) found a higher content of triglycerides in the serum of patients with coronary-artery disease than in that of normal subjects, while Böttcher et al. (1960 (b)) presented evidence of a strikingly high proportion of triglycerides in the coronary-artery lipids as compared with the aortic lipids. Hallgren et al. (1960) discovered a high level of plasma lipids, mainly triglycerides, in some of a group of seven diabetics with vascular complications, the fatty-acid composition of the triglycerides, cholesterol esters, and phospholipids being virtually

identical with that in seven controls. SCHRADE et al. (1961), examining healthy subjects, atherosclerotic patients and patients with idiopathic hyperlipidaemia, likewise found that with the rise of the blood lipids the rise in the percentage of glycerides is more marked than that of cholesterol esters and of phospholipids; on the other hand their work does bear evidence of changes in the fatty-acid composition of the various fractions.

In fact for a while research has been so intent on the lipids of the blood that it evoked the statement (DUFF, as cited by HAIMOVICI and MAIER 1961) that 'the casual reader of recent literature might wonder whether some authors conceive of an atherosclerosis so independent of the substrate of the vessel wall that it may occur in the absence of the blood vessels themselves'. Meanwhile the pendulum is swinging back again to a consideration of the process in the arterial wall. Earlier investigators (e.g. WINDAUS 1910; SCHÖNHEIMER 1926; MEEKER and JOBLING 1934; HIRSCH and WEINHOUSE 1943; BUCK and ROSSITER 1951) seem to have been concerned with relating some lipids to age and others to atherosclerosis more than we are today. Their chemical studies have been taken up again, using modern methods of fractionation. Data indicating a higher ratio of free to esterified cholesterol in atherosclerotic vessels were confirmed; likewise an increasing proportion of phospholipids, mainly attributable to a rise of the sphingomyelin content (SMITH 1960; BÖTTCHER and VAN GENT 1961). The announcement by NOBLE et al. (1957) that on chemical investigation of the earliest visible lesions (referring to 'thickened white-yellow areas') no significant elevation of the lipids could be found, is rather unexpected in view of the fact that for some decades numerous histopathologists have claimed otherwise, with rare exceptions, like MOON and RINEHART (1952) who described early lesions in which lipid deposition appeared to be absent.

The early lesions of the aorta have through the years attracted a great deal of attention, since minute fatty spots are already met with in the aorta of the very young child. MINKOWSKI (1947) saw them as the sequel of infectious disease, but most authors (KLOTZ and MANNING 1911; ZINSERLING 1923; KUBE and SSOLOWJEW 1930; ALBERT 1939) go out of their way to state they have not noticed any association with infectious disease and have come to the conclusion that there exists no difference between these fatty spots and the first atherosclerotic lesions (MOON 1957, GEER et al. 1961). In the opinion of DUFF and MCMILLAN (1951) and CRAWFORD (1961 (a)) the fatty spots observed

in the young aorta may either develop or retrogress. In comparing the conclusions of different morphologists one must be aware that the meaning of the terms 'early', 'first' and 'initial' may vary from author to author.

Crawford (1961 (a)) has given an up-to-date and extensive survey of morphological aspects in the pathogenesis of atherosclerosis. Refinement of well-tried histochemical methods (More, Movat and Haust 1957) and the application of modern techniques, such as electron-microscopy (Levene 1955) and fluorescence immunohistochemistry (Gitlin et al. 1957; Woolf and Crawford 1960) have supplied corroborative evidence of the presence of fibrin in even the smallest of 'atherosclerotic' lesions. Thus Rokitansky's supposition, propitiously revived by Duguid, has been substantiated.

How the simultaneous presence of the two elements, lipid and fibrin, in obstructive lesions can be accounted for must as yet remain speculative. In any case, a renewed inspection of the vascularization of the arterial wall, especially of the diseased arterial wall, seems opportune. Histopathologists have demonstrated most elegantly that the pathological intima and inner media become vascularized, whereas under normal circumstances they are not (Paterson 1936/38; Winternitz 1937; Horn and Finkelstein 1940; Geiringer 1951). Due to their pathological quality these capillaries would rupture easily, resulting in intimal haemorrhage. Some have seized upon this to explain the presence of lipids as due to the breaking up of erythrocytes. Some authors (Morgan 1956; Paterson 1957) report that fatty streaks are already vascularized, while others maintain that vascularization is characteristic only of far-advanced atherosclerosis, so that the presence of lipid in the early stages would not be explicable by this mechanism. Needless to say, it is but a single step from the presence of capillaries, especially of abnormal structure, to the origination of fibrin.

The status quo reached in morphological studies of the occlusion of arteries may well be conceived of as the point of contact of two lines of approach to the disease, for not only has attention been focussed on the relationship diet-lipids-atherosclerosis, but also on the relationship diet-lipids-thrombosis. Clinical textbooks as a rule tell of acute coronary-artery occlusion 'after a fatty meal'. Numerous authors present data of a shortening of the clotting time in postprandial hyperlipaemic plasma (e.g. Duncan and Waldron 1949; Waldron and Nichols 1952; Fullerton et al. 1953; Buzina and Keys 1956). Mustard

(1958) compared the effect of alimentary hyperlipaemia on blood clotting in atherosclerotic subjects with that in subjects with no clinical evidence of atherosclerosis. Pomerantz and Beatty (1953), and McDonald and Edgill (1957/59) reported that the blood of patients with ischaemia displayed a greater tendency to clot than the blood of healthy subjects. Spittel et al. (1960) used a modification of the thromboplastin generation test and established an accelerated clotting of the blood of patients with arterial and venous occlusions. Various lipid components have been tested for their effect on clot formation (Keys et al. 1957; Kommerell and Berger 1958). Pilkington (1957), studying the effect of fatty acids on recalcified plasma, and Connor and Poole (1961), on thrombosis-in-vitro, come to the conclusion that activity is connected with the saturated fatty acids of chain length C_{16} or more and that unsaturated fatty acids have no effect in vitro. At first sight it would seem likely that the haemophiliac is spared occlusive vascular disease. That this is not so (Astrup 1957; Stewart and Acheson 1957; Borchgrevink 1959; Loeliger et al. 1960) underlines the fact that a defect in the coagulation mechanism need not necessarily exclude the occurrence of obstructive lesions in the arteries. The question of the mucopolysaccharide heparin, which is an anticoagulant is intriguing. It has been shown that the mast cells, which are present along the bloodvessel lining, are capable of producing this substance (Florey 1955). Hahn (1943) discovered it to be capable of clearing hyperlipaemic plasma, in vivo. It has now become known that this action of heparin is due to the production of a clearing factor, a lipoprotein lipase which splits off the triglyceride moiety of chylomicrons and low-density lipoproteins (Korn, as cited by Kern 1961). Constantinides et al. (1953/56) succeeded in retarding the development of experimental atherosclerotic lesions (rabbit) by heparin injections. Graham, Lyon and Gofman presented data to this effect in 1951. Horita and Loomis (1954) achieved retardation of the formation of lesions in cholesterol-fed chickens not only with heparin, but also with dicoumarol, which is an anticoagulant but has no antilipaemic effect. Graham et al. also demonstrated a shifting of the higher Sf-lipoproteins to a lower group and observed a dramatic relief of angina pectoris on injection of heparin. The last point was confirmed by Kuo and Joyner (1957). Soffer and Murray (1954) concluded that in patients with idiopathic hyperlipaemia the injection of heparin caused a marked fall in the total lipid content of the blood, whereas Mitchell and Bronte-Stewart (1959) reported that the

post-heparin clearing rate in a group of ischaemic patients did not differ significantly from that in age-matched controls.

Various investigators (e.g. Astrup 1956) assume that under physiological conditions an equilibrium exists between the processes of coagulation and fibrinolysis. Accordingly shortcomings of the fibrinolytic system might lead to preponderance of the coagulative system and so contribute indirectly to the formation of thrombi. Fibrinolytic activity of the blood in patients with occlusive vascular disease was found to be significantly lower as compared with that of controls (Nestel 1959; Czarniecki 1960; Sarkar 1961). Nestel and Sarkar also confirmed that this phenomenon is in some way related to alterations of the β-lipoproteins, a connexion already pointed out by Greig and Runde in 1957. Greig had previously (1956) reported that alimentary hyperlipaemia has an inhibiting effect on fibrinolysis, and that feeding unsaturated fatty acids has not. Scott and Thomas (1957) compared the influence of various fats on fibrinolysis in rabbits and concluded that butter decreased the fibrinolytic capacity of the blood, while corn-oil appeared to have no influence at all.

§ 1.2 LIPIDS ASSOCIATED WITH POTENTIAL THROMBOPLASTIC ACTIVITY

Chargaff, Moore and Bendich (1942) isolated a macromolecular lipoprotein from saline extracts of bovine lung tissue which displayed striking thromboplastic properties. Subsequent investigations have laid the accent on the protein component, the exact nature of which has not been identified as yet. Neither has the structure of the lipid compound which constitutes the prosthetic group of the lipoprotein been established.

Of the free lipids (i.e. not in the form of lipoprotein complexes) which have been examined, the phospholipids in particular have become associated with thromboplastic properties. Poole (1958 (a)) reported that triglycerides and cholesterol have no effect on the coagulation mechanism. We* have checked the thromboplastic potentiality of the whole range of aortic lipid fractions, in work preliminary to the present investigation, and found that non of the non-phospholipid fractions had any measurable effect on coagulation in vitro.

* van Gent C. M., ter Haar Romeny-Wachter C. Ch.: unpublished observations.

While therefore the importance of phospholipids as a group in promoting clot formation is generally recognized, there is still much controversy as to which specific phospholipid fraction is responsible for thromboplastic properties. Results reported by various authors, using different methods of separating the phospholipids and of exploring their thromboplastic activity, are assembled in Table 1.
No investigations of the thromboplastic activity of phospholipids derived from the arterial wall have to our knowledge been reported.

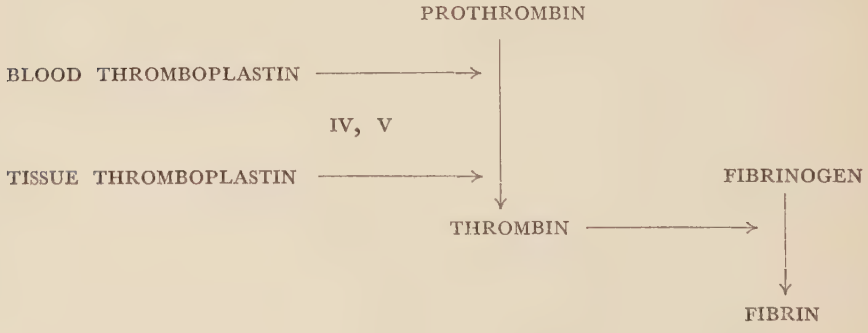
§ 1.3 THROMBOPLASTIC ACTIVITY OF AQUEOUS EXTRACTS OF ARTERIAL TISSUE

A relatively small number of authors have reported on this subject. The only factor the investigations have in common is that they employed aortas, obtained at autopsy, and that the tissue was suspended in saline.
WITTE and BRESSEL (1958) did not distinguish between atherosclerotic and normal aortas, at least they make no mention of it. They detected no difference between the activity of intima and that of the vessel wall as a whole. Their suspensions were tested on recalcified, (stocked) oxalated plasma and showed an activity of 5 to 80 % (average 22.9 %) of that of a standard thrombokinase.
ASTRUP et al. (1959) examined diseased and non-diseased material apart and attempted to separate the aortic wall in its respective layers: endothelium, intima, media and adventitia. Fresh, citrated, rabbit plasma served as coagulation substrate in their recalcification assays. Whereas the adventitia showed little or no thromboplastic activity, the tissue suspensions of the other layers possessed a high activity, especially the endothelial and intimal layers. The results in atherosclerotic and normal aortas were similar.
LOELIGER et al. (1960) mention finding marked thromboplastic activity in the intimal layer of the very young aorta, applied in saline to Quick's one-stage test.
STEVENSON et al. (1961) included the thromboplastin generation test in their assay methods and submit evidence that tissue suspensions of atherosclerotic vessels possess higher thromboplastic activity than those of normal ones, while in both groups the activity present in homogenates of the media is less than that of homogenates of the intima. Application of the one-stage prothrombin time determination

gave variable results: clotting times varying from 14 to 85 seconds, with an average of 40 seconds for the group of normal, and 41 seconds for the group of severely diseased aortas.
PERLICK reported at the Fourth International Angiological Congress (Prague, September 1961) a decline of tissue thromboplastic activity with progressively more severe atherosclerosis. DONNER at the same congress reported that aortas with ulcerated lesions had lower thromboplastic activity than those in which ulceration was absent.

§ 1.4 THE POSSIBLE RÔLE OF THE TISSUES IN THROMBOGENESIS

To describe at length how various schemes explaining the mechanism of blood coagulation have been devised, overhauled and reconstructed (see, for example, SEEGERS 1956; BIGGS and MACFARLANE 1957 (a); DEUTSCH 1959) lies outside the scope of this dissertation. We are concerned with only one of many aspects of coagulation here: the occurrence of a localized coagulative process at the vascular wall. In thrombogenesis at least two elements appear to be of importance beside the factors operating in the blood: contact with a 'foreign' surface and the liberation of a substance from the tissues.
In recent years the idea has gained ground that the coagulation mechanism of the blood makes use of two independent pathways, both of which ultimately lead to the formation of a thromboplastin. The thromboplastin reacts with the precursor of thrombin, prothrombin (= factor II) in the presence of calcium (= factor IV) and of factor V; thrombin, in its turn, accomplishes the change of fibrinogen (factor I) to fibrin, which is the essential component of every coagulum, be it in vivo (thrombus) or in vitro (clot).



This conception of a dual approach to the conversion of prothrombin involves one system, called *intrinsic*, in which 'blood thromboplastin' formation results from the interaction of the contact factors (XI and XII), the antihaemophilic factors (VIII and IX) and platelet factor; in the other system, the *extrinsic*, tissue factor and factor VII are required for the formation of 'tissue thromboplastin'; factors IV and X are common to both systems. This strict distinction resulted from observations on patients with various defects of coagulation and from the exploration of the effects the blood of these patients has on coagulation in vitro.

Various authors (e.g. Quick 1956; Biggs and MacFarlane 1957; Astrup et al. 1959; Loeliger et al. 1960) have pointed out that injured tissue might be a source of the tissue factor which is at the root of the extrinsic system; for reasons which are not yet clear, no intravascular thrombus formation takes place when there is a living, intact endothelial lining. If one is concerned with the possible rôle of thrombogenesis in occlusive vascular disease, this assumption has its attractions, although it is perplexing how tissue injury can be conceived of as something quite distinct from the possibility of 'foreign' surface contact. It seems evident that any kind of injury to the vessel wall would of necessity imply the existence of a breakdown, however small, in the endothelial barrier. The idea that the tissue factor is involved only in the extrinsic system, and the contact factor(s) only in the intrinsic, the two being virtually independent, has in the light of recent work lost some of its rigidity.

In 1957 Waaler demonstrated that tissue extract is capable of initiating the extrinsic as well as the intrinsic system in normal plasma, in which case the extrinsic system, which operates faster, determines the moment of clotting; when plasma lacking factor VII was used as a substrate, supposedly blocking the extrinsic pathway, clotting took place nevertheless as the result of the activation by tissue factor of the intrinsic system (which as mentioned before functions without factor VII). The intrinsic system also predominated when a weak tissue factor was applied.

Johnston and Hjort (1961) stated that during spontaneous coagulation of the blood, where tissue thromboplastin is absent, factor VII is activated by the interaction of factors XI and XII, VIII and IX and factor IV, again indicating an interplay between the two systems. Soulier and Prou-Wartelle (1960) presented evidence that it is factor XII which is activated by the contact with a 'foreign surface',

the resulting product reacting with factor XI to form a 'third prothromboplastic factor'.

Biggs and Nossel (1961) were persuaded to look further into the matter of separate pathways in the blood coagulation mechanism by the following considerations. In certain disorders of coagulation the defects in one of the two systems is not compensated for by the normality of the other system, (as in haemophilia = deficiency of factor VIII or IX, or in hypoproconvertinaemia = deficiency of factor VII). Furthermore in certain other disorders (as in a deficiency of factor XI or XII) the severe derangement of coagulation as projected in vitro cannot be reconciled with the relatively mild symptoms these deficiencies lead to. These authors come to the conclusion that to a certain extent the liberation of tissue extracts may initiate the contact phase of coagulation at the site of injury.

The publications mentioned above refer for the major part to the chain of events in normal haemostasis, which is again another aspect of coagulation. Little is known about the comparability of haemostasis and thrombogenesis (Poole and French 1961). On the other hand we are not disinclined to believe that a tissue factor which is released by the vascular wall at the site of a rupture might just as well be released by the vascular wall at the site of an atherosclerotic lesion or of a trauma resulting from the haemodynamic conditions. As already stated, we cannot conceive of an injury of the vascular wall which would not inevitably offer a 'foreign' surface to the blood stream; this certainly holds for the diseased vessel wall.

In summing up we come to the conclusion that only the slightest provocation is needed to set off a chain of reactions and interactions at the vessel wall leading to the formation of a thrombus. While the effect may be achieved by the intrinsic and extrinsic system independently, it appears from the above-mentioned publications that even if only one system is triggered, the other may also be set in motion.

CHAPTER II

MATERIALS AND METHODS

§ 2.1 EQUIPMENT

Portable vacuum flask (5L) for the transfer of material from the operating room at +2°C.
Glass plate, for dissecting.
Microscopical scissors, straight and curved.
Scalpel: a holder with interchangeable blades.
Microscopical forceps, pointed and obtuse.
Dissection microscope, magnification 6—50 × (Wild, Switzerland).
Refrigerator (operating at +2 °C).
Deepfreezer (operating at —30 °C).
Apparatus for freeze-drying.
Analytical balance.
Watchglasses, small and average sizes.
Potter homogenizers (5 and 20 ml); the apparatus consists of a glass test tube and a close-fitting power-driven pestle.
Variable voltage transformer.
Needles, of polished V2A steel, diameter 1.5 mm.
Transparent tubes, 500 × 5 mm, of flexible polyethylene, which at one end can be fitted to the needle.
Haemostatic forceps (Mosquito-type), to clamp the polyethylene tube at the desired moment.
Centrifuge, table model, operated at room temperature (Martin Christ, Germany, type UJOO).
Waterbath: a glass tank (40 cm long, 28 cm wide and 28 cm high) filled with distilled water which is electrically heated and stirred and thermostatically controlled at +37 °C.
Copper rack, for test tubes, which fits the waterbath approximately 4 cm under the water level.
Thrombelastograph.
Stopwatches (4).
Kolle-hook: L-shaped platinum or stainless steel hook (screwed into a 25 cm long holder), for establishing the moment of clotting.
Rubber suction-balloon for the controlled filling of pipettes.

Glassware:
Volumetric flasks (25 and 50 ml).
Centrifuge tubes, pyrex, (13—16 × 100 mm).
Test tubes of ordinary glass, round-bottomed, (16—17.5 × 100 mm).
Pipettes: graduated 10, 1 and 0.1 ml pipettes;
volumetric 0.1 and 0.05 ml pipettes.
Separating funnels.
Extraction flasks.
Soxhlet extraction apparatus.
Chromatography column, with glass filter and tap, (2 × 33 cm).
Chromatography column, with tap, (3 × 26 cm).
Equipment for dialysis of lipids through rubber membrane.
Waterbath for the evaporation of samples under nitrogen.
Magnetic stirrers.
Parafilm, to seal tubes (Marathon Corp., Menasha, Wisc., U.S.A.).

§ 2.2 REAGENTS AND COAGULATION SUBSTRATES

Methanol.
Chloroform.
Hydroquinone.
Cellulose powder, Whatman standard grade (W. and R. Balston Ltd., England).
Petroleum ether, b.p. 40 °—60 °C.
Petroleum ether, b.p. 80 °—100 °C.
Sea sand purified by acid and calcined (Merck, Germany).
Silicic acid 100 mesh (Mallinckrodt, New York).
Aqua bidestillata.
Saline: 0.9 % solution of NaCl.
Veronal buffer (Michaelis): 9.714 sodium acetate trihydrate and 14.714 g sodium diethylbarbiturate are dissolved in CO_2-free distilled water and the solution is made up to 500 ml. Of this stock solution 5 parts are mixed with 2 parts of a 8.5 % NaCl solution and the pH adjusted to 7.35 with x (about 4) parts 0.1 N HCl and 18 — x parts distilled water.
Sodium oxalate: 1.34 % solution of sodium oxalate in distilled water.
Sodium citrate: 3.8 % solution of disodium citrate in distilled water.
Homogenization solution: 2.7 ml of a 3.8 % sodium citrate solution are mixed with 60 ml Veronal buffer, to which is added 17.3 ml distilled water and 20 ml saline (pH 7.3).

Calcium chloride:

1.29 % (w/v) solution for recalcification of whole blood in thrombelastography.

1/40 M for recalcification of plasma.

1/30 M for recalcification of plasma plus homogenate.

$BaSO_4$ (pro roentgen), for adsorption.

Paraffin oil.

Silicone: 1167 A (Union Chimique Belgique), 4 % in distilled water for coating of glassware. (The vessel is immersed in the emulsion, allowed to drain off and subsequently baked in a dry sterilizing oven at 200 °C for four hours. The procedure is repeated when the glasswork has not been siliconized previously); S.F. 99 (General Electric, New York), 3 % in carbon tetrachloride for coating of needles. (After the needles have been immersed in the solution and drained off, they are baked in a dry sterilizing oven at 150 °C for eight hours.)

Collection of blood:

Blood is taken from the cubital vein with a sharp, siliconized needle (1.5 mm), to which a polyethylene tube of 50 cm length is attached. Venepuncture should be faultless. The first 2—3 ml are discarded after which the blood is allowed to flow freely through the tube into a silicone-coated volumetric flask containing the anticoagulant (one volume citrate or oxalate to nine volumes of blood). Care is taken that blood and anticoagulant are well mixed.
(Normal blood samples were collected from members of the personnel of the University Hospital, who volunteered to donate 50 ml 2—4 times a year.)

Normal citrated blood: this was used exclusively for thrombelastography. It was considered fit for use for three hours after venepuncture.

Normal platelet-poor oxalated plasma: the blood is poured into siliconized pyrex centrifuge tubes immediately after venepuncture has been completed and centrifuged at 3500 r.p.m. for 5 minutes. With a siliconized pipette the supernatant plasma is transferred to clean siliconized tubes and spun for 20 minutes at 3500 r.p.m. In order to obtain platelet-poor plasma it is imperative to avoid any disturbance of the partition level; this involves rejection of at least 5 mm of the plasma layer. (In checking this point by counting the platelets we have found that the manner in which the plasma is pipetted off is much more decisive for the number of platelets left in the plasma than is the speed with which it is centrifuged, although this should always exceed 3000 r.p.m.) The same precautions are taken after the plasma has been spun

the second time, when it is removed to siliconized test tubes placed in icewater. It is considered usable for five hours.

Normal serum: throughout the investigation serum was used from the same batch, from one donor. It was prepared by incubating the blood, in centrifuge tubes, for four hours at + 37 °C, after which it was centrifuged at 2000 r.p.m. for 10 minutes. The supernatant serum was then stocked in small portions, at —30 °C.

$BaSO_4$-adsorbed plasma: 100 mg $BaSO_4$ are transferred to a glass test tube and 1 ml freshly prepared oxalated plasma is added. The constituents are thoroughly mixed by agitating the tube for 5 minutes. After 3 minutes centrifugation at 2000 r.p.m. the supernatant is poured off and centrifuged again for 3 minutes at 2000 r.p.m. The BaSO-adsorbed plasma is poured off and put on ice; when tested, in a one-stage prothrombin-time determination, it should not clot within 6 minutes.

Platelet-poor citrated factor VII-deficient plasma: this was obtained from a patient with a congenital lack of factor VII.

Cephalin: this was prepared according to Bell and Alton (1954). Throughout the investigation the 'cephalin' was taken from a single batch, stocked in small portions at —30 °C.

§ 2.3 ARTERIAL TISSUE INVESTIGATED

In defining what basic material would be most appropriate for our investigations of the atherosclerotic arterial wall, we decided on sections of aorta and bifurcation of the aorta removed at reconstructive operations for aorto-iliac occlusion. All patients (fifteen) from whom we obtained diseased sections of the large arteries were suffering from severe symptoms of ischaemia, intermittent claudication (walking distance 50—200 meter) being the main cause of their visit to the policlinic for peripheral vascular diseases. The patients present, therefore, a history of ischaemic pain which cannot possibly be misinterpreted, and which is also objectively confirmed and localised by inspection, by palpation of the arteries of the extremities, and by oscillography and aortography.

In searching for comparable material we stipulated that all possibility of the arteries in question being affected by occlusive arterial disease could be excluded. It is only to the arteries of very young children that this is applicable.

To obtain such material — aortas in our case — it was necessary to

revert to preparations from autopsies. Owing to the small dimensions of these arteries, the combination of several preparations proved unavoidable. In this way one group of aortas was assembled from 0 to 3 months old, a second and third group from 0 to 10 months old and a fourth group from 0 tot 21 months old. One aorta of a child 12 years old was investigated separately.

Admittedly material acquired at necropsies — which take place from 12 to 24 hours after death — is subject to doubt concerning the extent to which the investigation is affected by changes in the tissues post mortem. It is with this in mind that sections of aorta were also examined from two patients, 12 and 13 years old, who were treated surgically for coarctation of the aorta. We are aware of the hazards connected with this kind of pathological structure.

§ 2.4 PREPARATION OF THE TISSUE BEFORE EXTRACTION

ad § 3.3 Immediately after dissection of the operation-material was complete it was enclosed in a receptacle at + 2 °C. A specimen was put aside for further examination by the pathologist.

Where the operation consisted of an endarterectomy, the preparative work had already been carried out by the surgeon: in thrombo-endarterectomy the artery is cleaned of its atherosclerotic intima and of as much of its medial layer as appears to be diseased, so that we received an intima+part media preparation of a severely atherosclerotic nature. If the surgeon decides to insert a vascular prothesis, the diseased part of the artery may be excised in its entirety. In that case we cut open the vessel, and after a thorough inspection of the arterial wall, tracked down the level along which diseased and non-diseased tissue could be properly separated with a small scalpel and forceps. Such a procedure likewise results in a highly atherosclerotic intima+part media preparation.

Twelve reconstructive operations yielded in this way twelve preparations. All were marked by advanced atherosclerotic lesions (atheromata and fibrous plaques, and by secondary complications thereof (ulceration, thrombosis, haemorrhage, calcification), but the extent to which the surface was involved appeared to be variable. Without exception the material was prepared immediately following surgical removal, cut into small pieces, thereafter at once stored at —30 °C and freeze-dried within the next twenty-four hours.

ad § 3.1 Three more preparations derived from aorto-iliac obstructions were dealt with as described above, except for an additional attempt to dissect an intimal and a medial layer. In the first and third instances sufficient structure was left to render the experiment possible. In the second case, however, no structure was apparent except in one site where a few fibres could still be identified as belonging to the medial layer; these were segregated and designated as a media preparation. Thus each of these three reconstructive operations resulted in two preparations: in the first and third cases one of the intimal layer and one of the media, in the second case one of intima-plus-media composition and one of media. Unlike the former atherosclerotic preparations these four were not freeze-dried, but the fresh tissue was homogenized within an hour after its removal at operation.

ad § 3.4 From a total of 50 obductions of very young children the aorta was collected; in three instances this implied the abdominal aorta only, but in the remainder the complete aorta was available, from the aortic valves to the bifurcation. In all of these aortas fatty streaks or spots were absent, as judged by thorough inspection with the naked eye.

The aorta is stripped of its adventitial coat, thereby disposing of a lipid depot which we consider to be of a purely physiological nature, in no way instrumental in athero- or thrombogenesis. The aorta is sharply cut across, and the raw edge clamped and held in sharp focus under a binocular dissection microscope (low magnification). With some experience it is not too difficult to make a line of cleavage in the outer 1/3 part of the media with a small, sharply pointed scalpel (or with an injection needle). With two forceps the two layers are pulled apart: on one side the adventitia with the external elastic lamina, to be discarded, on the other side the intima with most of the media. Care is taken not to wipe or crush the intimal surface. In this manner 32 intima-plus-media preparations of non-atherosclerotic aortas were obtained.

The medial tissue was expressly included in this material, intended for comparison with the operation material: the latter undoubtedly contains medial elements as the advanced atherosclerotic lesion extends into the media.

As the investigations proceeded we became increasingly interested in exploring the thromboplastic potentiality of the intima and media separately. (This concurred with a same trend in the investigations

of the composition of the lipids of the arterial wall, by Böttcher and co-workers.) The aorta is spread out under the dissection microscope with the intimal surface in sharp focus. With a small, extremely sharp scalpel a shallow vertical cut is made, after which the thinnest possible layer is grasped with a not too large forceps and painstakingly torn off. Sometimes the upper layer is dissected this way in one manipulation, sometimes one has to start over and over again; we have not been able to detect any connexion between this and, to name a few possibilities, the lapse of time between death and necropsy, age, sex, cause of death or the administration of drugs. The notion that in the first, 'easy', case one is lucky enough to have succeeded right away in severing the layers along the borderline between intima and media, i.e. the internal elastic lamina, while in the second, 'difficult', case one is forced repeatedly to grope for this line of cleavage is obvious but fallacious. As in the case of stripping off the adventitia, which proved to be satisfactory, we have checked the dissection of intima and media by means of histochemical methods. A slide is made of a specimen in which both severed and unsevered tissue are represented, and the internal elastic lamina is made visible with a specific elastic tissue stain. So far we have not been able to discover in these samples any correlation between the subjective judgment of the success of the dissection and the objectively judged results thereof. It therefore appears that we have not succeeded in developing a method of separation of intima and media which is truly reliable and reproducible. For this reason we like to name the product of forceps and scalpel 'intimal layer' rather than 'intima'; the designation 'media' is effectual, however, as the specific fibrous aspect of this tissue is easily identified at the (low) magnification of the dissection microscope.
18 aortas were thus separated into intimal layer and media preparations.

ad § 3.2 One aorta, of a child which succumbed at twelve years of age, was separated as described above. The resulting intimal layer preparation and media preparation were homogenized within an hour after being removed at autopsy, which took place 12 hours after death. This was also the procedure followed with the preparations resulting from two cases (12 and 13 years old) of surgical treatment of coarctation of the aorta. Coarctation of the aorta is an anomaly, existing from birth. The operation consists of resecting the stenosed part of the aorta, including, for practical reasons, a non-stenosed margin. It is

known that the aortic wall approximating a coarctation may well display alterations of an atherosclerotic nature, but these appear to be not nearly so pronounced in younger individuals. This encouraged us to examine the marginal tissue, especially as it seemed to be our only hope of obtaining young arterial tissue in a fresh state. The specimen was carefully cut open and thoroughly inspected before discarding the stenosed part. In the first case the remnant measured approximately 2 × 9 mm, in the second case it was even less.
Here again the thinnest possible layer was dissected from the intimal surface, followed by stripping off the adventitial tissue. In neither section were difficulties encountered with the preparation. To the naked eye the prepared tissue appeared to be non-diseased; owing to the scarcity of tissue no histochemical control was undertaken.
In this way we obtained two relatively non-atherosclerotic preparations of the intimal layer and two non-atherosclerotic preparations of the media.

Fig. 1. *Homogenizer* (POTTER).

§ 2.5 AQUEOUS EXTRACTION

The preparation is weighed on the analytical balance and quantitatively transferred to the glass test tube of a Potter homogenizer (fig. 1), with the homogenization medium as a vehicle in a proportion of 2 ml per 100 mg tissue.
The left hand holds the test tube fast, moving it rapidly up and down, while the right hand is free to adjust the power by which the glass pestle is rotated: in moving the tube downwards it is necessary to lessen the speed. After approximately 5 minutes, an opaque suspension is obtained which is put away in the dark at +2 °C for 24 hours to settle. (All preparations which were treated in this way were homogenized within an hour after their removal at operation or autopsy.) After

24 hours the supernatant is poured off and immediately tested for its thromboplastic properties.

§ 2.6 NON-AQUEOUS EXTRACTION AND SEPARATION OF THE LIPIDS

For completeness sake a detailed description is presented of the extraction and separation of the lipids, which was performed by other members of the atherosclerosis-research team.

Extraction of the lipids begins at the moment when the material is stored in the freeze-dried state under methanol-chloroform (1 : 2, v/v) at —30 °C. Former investigations have demonstrated that the lipids suffer no changes when they are stored under methanol-chloroform at —30 °C for 9 months, and probably for a much longer period.

When taken out of storage the tissue is filtered off and stirred at room temperature with 200 ml methanol-chloroform for 3 hours, using a magnetic stirrer. The tissue is again filtered off and re-extracted with 200 ml fresh methanol-chloroform for 24 hours. This procedure is repeated, after which extraction is completed by 3 hours' Soxhlet extraction with methanol-chloroform to which is added an antioxidant (1 mg hydroquinone). The extracts (five in all) are then combined and evaporated to a small volume.

Separation of the lipids from non-lipids consists of purification on a cellulose column: powdered cellulose is made into a slurry with methanol-chloroform and packed into a column 2 × 30 cm. It is washed with the solvent until no further material is eluted. The lipoid extract is applied to this column in not more than 5 ml solvent, and the lipids are eluted with 100 ml methanol-chloroform. Amino-acids and other non-lipids travel down the column at a much lower rate and remain behind on the cellulose. The eluate is evaporated almost to dryness, care being taken to remove the final traces of methanol by adding small portions of petroleum ether (bp. 60—80°) and evaporating almost to dryness twice more. The lipids are then dissolved in about 25 ml petroleum ether (bp. 40—60°) and transferred to a rubber dialysis bag.

For the separation of the phospholipids from the total lipid use was made of the circumstance that in the non-polar solvent the phospholipids form micelles (consisting of about 70 molecules) which are too large to pass through the rubber membrane. The membrane, which also contains the material to be dialysed, consists of a finger of a surgical glove, which is first extracted with ether, then with petroleum ether, each for 3 hours in a Soxhlet apparatus. The 2-cm glass tube, which supports the rubber finger, secured to it by a nichrome resistance wire, also acts as a guide for an electrically rotated, shaped glass rod which stirs the contents of the bag and, by pushing against the membrane, agitates the dialysate also. The outer vessel has a constant level device allowing continuous renewal of solvent and collection of dialysate (at 1 drop every 2—3 seconds). Twenty-four hours after dialysis has been started the receiver is changed, after which hourly checks are made for further material appearing in the dialysate: when this does not exceed 0.2 mg, dialysis is stopped. The dialysate, which contains free fatty acids, free cholesterol, cholesterol esters and triglycerides is evaporated to a smaller volume and stored at —30 °C after 10 % has been pipetted off, evaporated to dryness and weighed. The undialysable part of the lipids, containing phospholi-

pids, is quantitatively transferred from the dialysis bag with petroleum ether and methanol-chloroform; after 10 % has been pipetted off, evaporated, weighed and then stored under methanol-chloroform at —30 °C, the remainder is evaporated to dryness and taken up in about 5 ml chloroform.
Subsequently this phospholipid mixture is subjected to chromatography on a silicic acid column: Mallinkrodt silicic acid, 100 mesh (14 g) is made up as a slurry in methanol-chloroform (1 : 1, v/v) into a short, wide column (3 cm in diameter and 3,5 cm high). A ring of filter paper and 0,5 g of glass helices on top of the column prevent disturbance of the surface during additions. The adsorbent is activated by passing 200 ml of methanol-chloroform (1 : 1, v/v) through it, followed by 200 ml chloroform to remove the methanol. The phospholipid mixture, up to a maximum of 200 mg, is applied to this silicic-acid column. To remove any lipids, other than phospholipids, remaining after the dialysis, a preliminary elution with 100 ml chloroform is carried out. This is followed by discontinue elution with stepwise increasing proportions of methanol in chloroform. Elution
with 100 ml methanol-chloroform 1 : 7 (12½ % MC) yields a first group, elution
with 100 ml methanol-chloroform 1 : 4 (20 % MC) a second group of choline-free phospholipids (so-called cephalins).
200 ml methanol-chloroform 1 : 1 (50 % MC) elutes the lecithins, plasmalogens related to the lecithins, lyso-cephalins and part of the sphingomyelins.
200 ml methanol-chloroform 4 : 1 (80 % MC) to which 4 vol. % water is added, elutes the remainder of the sphingomyelins and lecithins.
After five fractions have been collected, evaporated to dryness and weighed, they are suspended in a Veronal buffer (pH 7.35) in a concentration of 1 mg per ml. The suspensions are stored at —30 °C to await testing for thromboplastic activity.

The method described above for the separation of the lipids from the non-lipids is not practicable in those instances where the fresh tissue has not been freeze-dried but has been extracted with an aqueous solution in order to obtain an impression of the thromboplastic activity of the tissue as a whole. In those cases (§ 3.1.) the method of Folch and collaborators was applied, in which the extract is partitioned between the two layers resulting from the mixture methanol, chloroform and water in the proportions 4 : 8 : 3. The lipids select the chloroform layer while the non-lipids migrate to the prodominantly aqueous layer. The chloroform layer can be collected from the separating funnel in which the mixture is allowed to stand overnight. To obtain a clear solution centrifuging of the aqueous layer is necessary.

§ 2.7. ASSAYS OF THROMBOPLASTIC ACTIVITY

From the need of a better understanding of certain disorders of haemostasis various tests have arisen which reflect the interplay of blood clotting factors. In these assays it is customary to keep all factors, including those representing thromboplastic activity, constant while the plasma or the serum of the patient is the uncertain element. In the present investigation we have tried three of these methods, in which we substituted our suspensions for the factors representing thromboplastic activity, while striving to keep all other factors constant.
The following coagulation assays were applied:

The thromboplastin generation test (according to BIGGS and DOUGLAS, 1953) slightly modified.

In a glass test tube, placed in the waterbath (at +37 °C) in a copper rack, are mixed:

0.3 ml serum, diluted 1 : 10 with Veronal buffer, pre-incubated in a test tube of ordinary glass at room temperature for at least one hour, and for not more than two hours,

0.3 ml $BaSO_4$-adsorbed plasma, diluted 1 : 5 with Veronal buffer,

0.3 ml of the suspension to be investigated,

0.3 ml 1/40 M $CaCl_2$ (1/30 M $CaCl_2$ in some cases) is added, at which moment a stopwatch is started. From this so-called incubation mixture, in which thromboplastin forms, amounts of 0.1 ml are removed at intervals (2, 4, 6, 8, 10, 12 and 15 minutes) and added to aliquots of 0.1 ml normal oxalated plasma previously pipetted out and placed in the waterbath. Simultaneously with the addition of 0.1 ml of the incubation mixture, 0.1 ml 1/40 M $CaCl_2$ is blown into the tube containing the oxalated plasma. A second stopwatch is started at this moment and the time which elapses before a substantial clot can be pulled up with the Kolle-hook is recorded.

The prothrombin-time determination (QUICK's one-stage system, 1957)

0.1 ml oxalated plasma is transferred to a glass test tube, placed in a copper rack in the waterbath (at +37 °C), 0.1 ml of the suspension to be investigated is added to the plasma and the contents of the tube are mixed thoroughly by swirling. 60 seconds later 0.1 ml of 1/40 M $CaCl_2$ (1/30 M $CaCl_2$ in some cases, see 'Reagents') is blown out forcibly from a volumetric pipette into the test tube. A stopwatch is started at the exact moment the $CaCl_2$ is added and the tube is quickly swirled again. With the Kolle-hook the coagulation mixture is manoeuvred gently until a substantial clot can be pulled up, the instant at which this occurs being registered with the stopwatch. The coagulation-time is recorded.

Thrombelastography (HARTERT, 1951)

The thrombelastograph is an apparatus which records automatically changes in the viscosity of the blood during the various phases of clot formation. Of these the first onset of clotting, the 'reaction time' has received our attention in the present investigation.

From a torsion wire a steel rod is suspended of which the cylindrically shaped end hangs free of contact into a cuvette of polished V_2A steel

(one thrombelastograph has three cuvettes). The cuvette rotates periodically through 90 ° about a vertical axis, while the temperature of the apparatus is thermostatically maintained at +37 °C, (fig. 2). 0.5 ml of fresh citrated blood, which is kept at room temperature, is transferred to a siliconized glass tube in a rack in the waterbath (+37 °C). To this 0.05 ml of the suspension to be tested is added

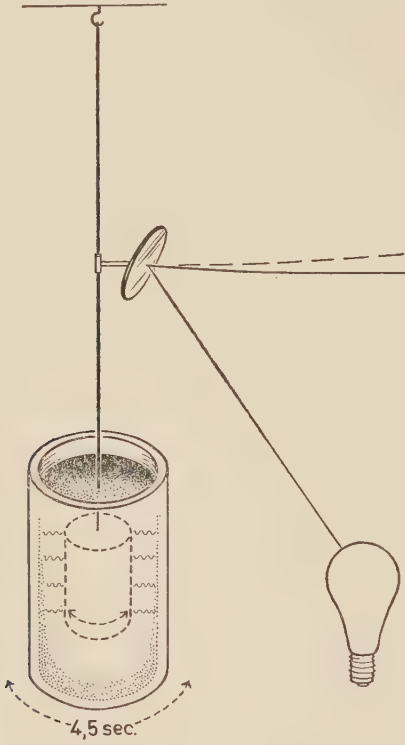


Fig. 2. Principle of thrombelastography.

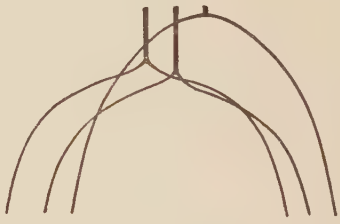


Fig. 3. Examples of thrombelastograms.

and the contents are well mixed. After 60 seconds 0.05 ml of a 1.29 % solution of $CaCl_2$ is blown from a volumetric pipette into the tube, at which moment a stopwatch is started. The tube is quickly swirled before the contents are poured into a cuvette of the thrombelastograph. The rod is lowered, superfluous blood is drawn off with a clean syringe and the contents of the cuvette are sealed off from air by means of paraffin oil. As long as the cuvette is filled with a substance which is completely liquid the rod remains motionless, but when a coagulum begins to form the rod starts to follow the movements

photographically on a slowly revolving film, which results in a thrombelastogram (fig. 3). From the thrombelastogram the time until the initiation of clot formation is deduced which, added to the time elapsed between the addition of the $CaCl_2$ and the moment the film is started, is recorded as the reaction time.

§ 2.8 EXPRESSION OF THE RESULTS

Thromboplastin Generation Test

In this two-stage coagulation assay the amount of thromboplastin generated is gauged by adding aliquots of the incubation mixture at intervals to normal plasma and recording the clotting times. The shortest of these represents the thromboplastic potentiality of the tissue factor investigated.

The inconstancy of normal plasma, which shows substantial individual variations has to be allowed for. Therefore from the plasma of each donor a thromboplastin generation test was performed with a standard 'cephalin' suspension. With the standard 'cephalin' used the clotting time averaged 10.4 seconds ($\pm$ 1.56 sec.). Whenever the clotting time found with the standard 'cephalin' was shorter than 10.4 seconds, the difference was added to, whenever it was longer than 10.4 seconds, it was subtracted from the clotting time registered for the tissue factor investigated.

In order to relate clotting time and concentration of thromboplastin a thromboplastin dilution curve was made (Biggs and MacFarlane 1957), using the plasma of a number of donors (with standard 'cephalin' in the incubation mixture). A range of 7 to 200 seconds was needed in order to cover clotting times found in the tissue factor investigations. Only from 7 to 15 seconds the plot of the log concentration against clotting times is straight (fig. 4). Quite arbitrarily 10 seconds was taken to represent a '100 %' thromboplastin concentration.

It proved to be hazardous from a statistical point of view to plot a curve for a thromboplastin concentration lower than '30 %', the individual variation in this clotting range being much greater. For this reason clotting times of 15 seconds and longer had to be expressed as follows:

from 15 to 23 sec. (S.D. $\pm$ 3 sec.): 20—30 % thromboplastin conc.
from 23 to 45 sec. (S.D. $\pm$ 6.5 sec.): 10—20 % thromboplastin conc.
from 45 to 92 sec. (S.D. $\pm$ 13.8 sec.): 2—10 % thromboplastin conc.

It must be pointed out that these designations are only of formal and comparative significance.
Substitution of Veronal buffer for tissue factor in this test yields an apparent thromboplastin concentration of between 2 and 20 %.

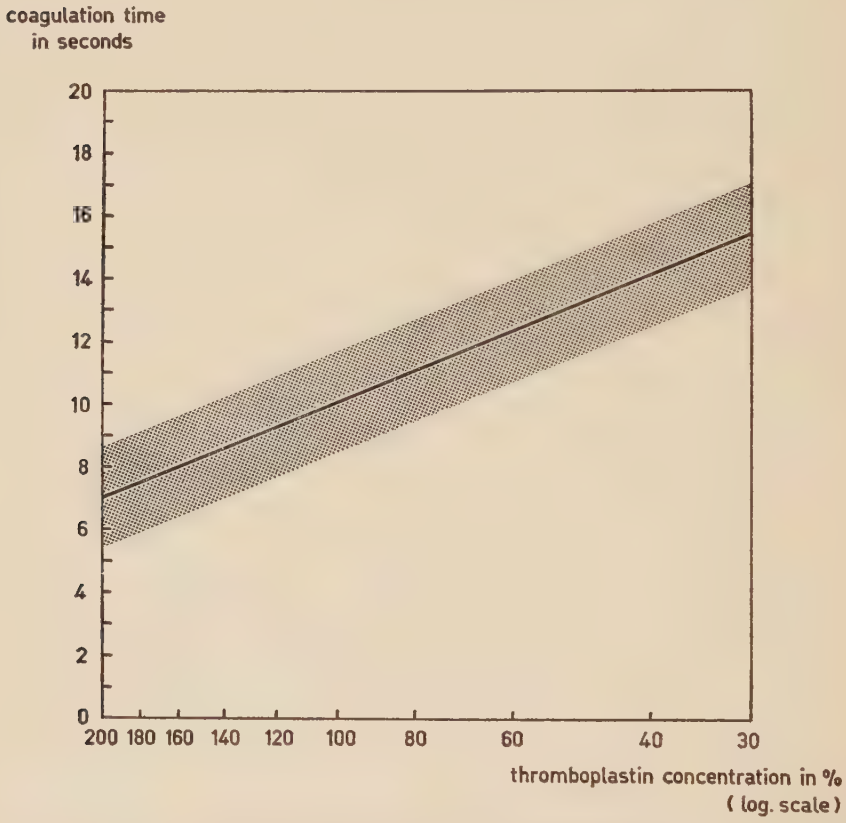


Fig. 4. Relation between thromboplastin concentration and clotting time. The chance that a given determination in this region will fall in the area indicated by the grey band is > 95 %.

Prothrombin-time determination (Quick's one-stage system)

The determination was performed in duplicate. The value given is the average of the two clotting times. The 'control' is the time recorded when instead of the tissue factor, the medium in which this was suspended was applied to the test system.

Thrombelastography

A determination in duplicate was performed in most cases; the citrated fresh blood was considered unfit for further use in this test three hours after venepuncture.

Whenever two reaction times were recorded both are presented, in order to give some indication of the reproducibility of the assay method.

Here again 'control' refers to the reaction time obtained when instead of the tissue factor the medium in which this was suspended was added to the blood. The average reaction time of all our 'controls' was 7 ($\pm$ 1 2/5) minutes.

CHAPTER III

RESULTS

§ 3.1 THROMBOPLASTIC ACTIVITY OF THE ATHEROSCLEROTIC SECTIONS OF THE BIFURCATION OF THREE AORTAS

In three aortic sections which were removed at operation because of clinically manifest narrowing of the bifurcation of the aorta, dissection of an intimal layer was attempted. The results of such an undertaking in severely diseased material should be regarded with a maximum of reserve (see § 2.4). The preparations were homogenized within an hour after their removal at operation and tested 24 hours later (see § 2.5).

The results of the coagulation assays are given in Table 2. The homogenate from the intimal layer of preparation I showed 200 % activity in the thromboplastin generation test (TGT), which means that clotting times shorter than 7 seconds were recorded; the media of I displayed 100 % activity, corresponding to a clotting time of 10 seconds. Thromboplastic potentiality of preparation II, the extremely necrotic tissue which we named intima + media, as well as the medial tissue dissected in this case, showed 30 % activity, in other words clotting times approximating 15 seconds. The TGT of the intimal layer of preparation III showed 13 seconds to be the shortest clotting time here. The homogenate from the media of this preparation displayed, however, an inhibitory effect, a percentage lower than 2 %, meaning that clotting times longer than 92 seconds were registered.

The results of the prothrombin-time (PT) determination pointed in a similar direction, but they were not nearly so pronounced as in the TGT.

The coagulative influence of the homogenates of preparations I and II on recalcified citrated blood, as recorded with the thrombelastograph, was manifest.

In order to obtain an impression of the nature of the substance associated with potential thromboplastic activity, the homogenates of preparations I and II were treated according to FOLCH (see § 2.6), as far as the quantity of homogenate and tissue permitted.

Table 2. *Thromboplastic activity of the homogenates of atherosclerotic aortic tissue*

Preparation	Section of aorta	Concentration of suspension in test	Thromboplastin Generation Test (in %)	Prothrombin Time determination (in seconds)	Reaction Time in Thrombelastography (in minutes)
I	Intimal layer (70 mg)		(100 % = 10 sec.)	(control: 189 sec.)	(control: 7, $4\frac{3}{4}$ min.)
		100 %	> 200	39	$1\frac{1}{2}$, $1\frac{1}{4}$
		10 %	71	82	$1\frac{1}{2}$, $2\frac{3}{4}$
	Media (1304 mg)				
		100 %	100	48	$1\frac{1}{2}$, $1\frac{1}{2}$
		10 %	2 — 10	102	$2\frac{1}{4}$, $2\frac{3}{4}$
II	Intima + Media (7000 mg)		(100 % = 10 sec.)	(control: 169 sec.)	(control: $8\frac{1}{2}$, $7\frac{1}{2}$ min.)
		100 %	30	60	$2\frac{3}{4}$, 3
		10 %	10 — 20	105	4 , $4\frac{1}{4}$
	Media (70 mg)				
		100 %	20 — 30	36	$1\frac{1}{4}$, $1\frac{1}{2}$
		10 %	20 — 30	77	$2\frac{1}{4}$, $2\frac{1}{2}$
III	Intimal layer (53 mg)		(100 % = 10 sec.)	(control: 165 sec.)	
		100 %	20 — 30	32	
		30 %	50		
		10 %	36	72	
	Media (1155 mg)				
		100 %	< 2	86	
		30 %	< 2		
		10 %	10 — 20	141	

Table 3. *Thromboplastic activity of preparation I after Folch-treatment*

	Concentration in test (γ/ml)	Thromboplastin Generation Test (in %)	Prothrombin Time determination (in seconds)	Reaction Time in Thrombelastography (in minutes)
Lipid fraction of:		(100 % = 10 sec.)	(Control: 226 sec.)	(Control: $6\frac{3}{4}$, 5 min.)
Intimal tissue	1000	2 — 10	179	5 , $4\frac{1}{4}$
	100	2 — 10	200	$4\frac{1}{2}$
Intimal homogenate	1000	2 — 10	187	$5\frac{1}{2}$, 4
	100	2 — 10	205	$4\frac{1}{2}$
Media	1000	2 — 10	198	$5\frac{1}{4}$, $3\frac{3}{4}$
	100	2 — 10	203	$4\frac{1}{4}$
Media homogenate	1000	2 — 10	163	6 , $4\frac{1}{2}$
	100	2 — 10	203	6
Non-lipid fraction of:				
	1000	< 2		
Intimal homogenate	100	2 — 10		
	1000	< 2		
Media homogenate	100	2 — 10		

Table 4. *Thromboplastic activity of the homogenate from preparation II (intima + media)* after Folch-treatment*

	Concentration in test (γ/ml)	Tromboplastin Generation Test (in %)
		(100 % = 10 sec.)
Lipid fraction		
	1000	2 — 10
	100	2 — 10
Non-lipid fraction		
	1000	2 — 10
	100	2 — 10

* Insufficient homogenate of the media was available for further treatment.

The resulting chloroform fraction, containing the lipids, and the aqueous fraction, containing the non-lipids, were subsequently tested for thromboplastic activity. The data obtained for preparation I are presented in Table 3, those for preparation II in Table 4. Both lipid and non-lipid fractions behaved indifferently, when applied in the various coagulation assays, clotting times in the range from 23—45 seconds being recorded.

A possible explanation of the lack of activity of the lipid fraction could be that the positive effect of one group of phospholipids might be counterbalanced by the negative effect of another group. The chloroform fraction of the homogenate of preparation II was therefore fractionated according to § 2.6; the four phospholipid fractions

Table 5. *Thromboplastic activity of the phospholipid fractions of the homogenate from preparation II (in %)*

Phospholipid fraction	Concentration of phospholipid in Thromboplastin Generation Test		
	1000 γ/ml	300 γ/ml	100 γ/ml
12½ % MC*	2 — 10	2 — 10	2 — 10
20 % MC	2 — 10	< 2	< 2
50 % MC	< 2	< 2	< 2
80 % MC	2 — 10	< 2	< 2

* MC = methanol-chloroform.

resulting did not display any thromboplastic activity, as is shown by the data in Table 5.

The substance responsible for the activity found in the homogenates may not be the only active principle in the arterial wall, for it is known that certain phospholipids form part of the structure of the tissues, and it is conceivable that they are not liberated during homogenization but remain bound to the tissue residue left behind when the homogenate is poured off. With this in mind the tissue residue from preparations I and II were extracted with methanol-chloroform (MC); four phospholipid fractions were prepared from the extract by chromatography (see § 2.6) and tested in the TGT (Table 6).
All fractions appeared to be indifferent (2—10 %) or inhibitory (< 2 %), except for the 20 % MC fraction of the media from preparation I, which displayed an activity of 20—30 %.

Table 6. *Thromboplastic activity of the phospholipid fractions of the tissue residue of preparations I and II (in %)*

Preparation	Phospholipid fractions	Concentration of phospholipid in Thromboplastin Generation Test		
		1000 γ/ml	300 γ/ml	100 γ/ml
I (Media)	12½ % MC*	< 2	< 2	< 2
	20 % MC	20 — 30	10 — 20	10 — 20
	50 % MC	2 — 10	2 — 10	2 — 10
	80 % MC	2 — 10	2 — 10	2 — 10
II (Intima + media)	12½ % MC	< 2	2 — 10	2 — 10
	20 % MC	< 2	< 2	< 2
	50 % MC	2 — 10	2 — 10	2 — 10
	80 % MC	< 2	< 2	< 2

* MC = methanol-chloroform.

§ 3.2 THROMBOPLASTIC ACTIVITY OF THE INTIMAL LAYER AND MEDIA OF THE RELATIVELY NON-ATHEROSCLEROTIC SECTIONS OF THREE AORTAS

It was mainly with the object of comparing the homogenates of fresh material and of that obtained at autopsy, that the investigations presented in this section were undertaken.
Preparation A (Table 7) concerns the aorta of a twelve year old girl, who died as the result of an accident. The obduction took place

Table 7. *Thromboplastic activity of the homogenates of relatively non-atherosclerotic aortic tissue*

Preparation	Source of preparation	Concentration of suspension in test	Thromboplastin Generation Test (in %)	Prothrombin Time determination (in seconds)	Reaction Time in Thrombelastography (in minutes)
A	*Aorta (autopsy)* (girl, aged 12)		(100 % = 10 sec.)	(control: 170 sec.)	(control: $5\frac{1}{4}$, $5\frac{1}{4}$ min.
	Intimal layer (100 mg)				
		100 %	10—20	60	$1\frac{1}{2}$, $1\frac{1}{2}$
		10 %	2—10	86	$2\frac{1}{2}$
	Media (100 mg)				
		100 %	< 2	no clotting	$4\frac{1}{4}$, $3\frac{3}{4}$
		10 %	< 2	185	$4\frac{1}{4}$
B	*Aorta (operation)* (boy, aged 12)		(100 % = 10 sec.)	(control: 205 sec.)	(control: $8\frac{1}{4}$, $8\frac{1}{4}$ min.)
	Intimal layer (5 mg)*				
		100 %	—	—	—
		10 %	87	58	$1\frac{1}{2}$
		1 %	20—30	—	$3\frac{1}{4}$
	Media (100 mg)				
		100 %	126	45	$1\frac{1}{2}$
		10 %	20—30	85	$4\frac{1}{4}$
C	*Aorta (operation)* (boy, aged 13)		(100 % = 10 sec.)	(control: 169 sec.)	(control: $8\frac{1}{2}$, $7\frac{1}{2}$ min.)
	Intimal layer (10 mg)				
		100 %	20—30	**	$1\frac{1}{4}$, $1\frac{1}{2}$
		10 %	20—30	52	$2\frac{1}{2}$, $2\frac{1}{4}$
	Media (80 mg)				
		100 %	20—30	36	$2\frac{1}{4}$, $2\frac{1}{2}$
		10 %	10—20	98	$3\frac{1}{2}$, $3\frac{1}{1}$

* As the usual concentration of the homogenate is 100 mg per 2 ml, the available 5 mg intimal layer homogenized with 1 ml suspension roughly corresponds to a 10 % suspension.

** Insufficient amount; P.T. of 50 % homogenate was 50 sec.

12 hours after death. On thorough macroscopical inspection the intimal surface of the aorta appeared normal, except for a few pinpoint sized white spots near the point of branching of some of the intercostal arteries. The preparation was homogenized within 13 hours after death and tested 24 hours later (see § 2.5).

Preparations B and C consisted of the least pathological margin of two aortic sections which were removed at operation because of coarction (see § 2.4). Both preparations were homogenized within an hour after removal at operation, and tested 24 hours later.

In the thromboplastin generation test (TGT) of preparation A the intimal-layer homogenate showed no thromboplastic potentiality, while the media homogenate had an inhibitory effect (it was repeatedly shown that no clotting resulted at 8 minutes' incubation time or longer). The findings in the media were reflected in the prothrombin-time (PT) determination. Clotting times as measured by the effect of the homogenate on recalcified citrated blood in the thrombelastograph are unexpectedly short, considering the results in the other two assays.

Of preparation B only 5 mg of intimal layer was available; this would have had to be homogenized with 0.1 ml medium in order to obtain the usual concentration (see § 2.5) for the '100 %' suspension. For practical reasons the 5 mg were homogenized with 1 ml, resulting in a suspension roughly corresponding to the '10 %' suspension of other samples. The thromboplastic activity of this suspension amounted to 87 % in the TGT, which means that clotting times of about 11 seconds were recorded; the media of preparation B also possessed thromboplastic activity (126 %).

Of preparation C the intimal layer and the media did not behave very differently in the TGT; both yielded an activity of 20—30 %, which corresponds to clotting times in the range of 15 to 23 seconds. Homogenates of both B and C, when tested in the PT determination (substrate: recalcified platelet-poor plasma) and by thrombelastography (substrate: recalcified citrated blood) displayed an accelerating influence as compared with the clot formation in the controls of these two assays.

§ 3.3 THROMBOPLASTIC ACTIVITY OF THE PHOSPHOLIPID FRACTIONS OF ATHEROSCLEROTIC SECTIONS OF THE BIFURCATION OF TWELVE AORTAS, OF TWO THROMBI AND OF A GROUP OF EXCISED ATHEROSCLEROTIC LESIONS

The first twelve preparations (Table 8) were removed at operation because of clinically manifest narrowing of the bifurcation of the aorta.

Preparations 7^{thr} and 12^{thr} were partly organized thrombi easily detachable from the aortic sections 7^{w} and 12^{w} respectively. Preparation 13 consisted of atherosclerotic lesions, which were excised from several severely diseased aortas obtained at autopsy.

Non-aqueous extraction and fractionation (see § 2.6) of these prep-

Table 8. *Thromboplastic activity of the 20% MC phospholipid fraction of diseased tissue (in %)*

No. Preparation	Concentration of phospholipid in Thromboplastin Generation Test				Further Observations
	1000 γ/ml	300 γ/ml	100 γ/ml	30 γ/ml	
1*	10 — 20	10 — 20	10 — 20	10 — 20	In this case for 20 % MC read: 33 % MC
2	46	104	81		
3	100	43	10 — 20		
4	158	120	74		Preparations removed at operation because of clinically manifest narrowing of the bifurcation of the aorta
5	63	93	89		
6	65	83	20 — 30		
7^{w}	80	138	47		
8*	10 — 20		2 — 10	10 — 20	
9	50	65	44		
10	2 — 10	20 — 30	10 — 20		
11	10 — 20	10 — 20	10 — 20	10 — 20	
12^{w}	81	87	41		
7^{thr}	146	87	20 — 30		Thrombus detached from prep. 7^{w}
12^{thr}	< 2	10 — 20	— 20		Thrombus detached from prep. 12^{w}
13	104	134	65		Atherosclerotic lesions (autopsy)

* In these cases phospholipids were separated from other lipids by silicic-acid chromatography instead of by dialysis.

arations yielded five phospholipid fractions each. The fraction eluted from the silicic acid with chloroform, the 50 % MC* and the 80 % MC fractions behaved indifferently (2—20 %) in the thromboplastin generation test (TGT). The $12\frac{1}{2}$ % MC fractions gave in general much longer clotting times, such as may be associated with an inhibitory effect (< 2 %).

Table 8 shows the results obtained with the 20 % MC fractions. Of the 20 % MC fractions from twelve atherosclerotic aortic sections eight displayed thromboplastic activity, in varying degree. One 20 % MC fraction of thrombus showed almost 150 % thromboplastic activity (clotting times approximating 10 seconds), while the corresponding fraction from the other thrombus did not show any activity at all. The combined atherosclerotic lesions possessed thromboplastic activity of more than 100 % in the 20 % MC fraction.

§ 3.4 THROMBOPLASTIC ACTIVITY OF THE PHOSPHOLIPID FRACTIONS OF FOUR GROUPS OF NON-ATHEROSCLEROTIC AORTAS

The aortas described in this section were obtained at autopsy. No atherosclerotic lesions were observed macroscopically in any of the preparations.

The aortas of group I and II (Table 9) were dissected within 6 hours of their removal at autopsy. Seven aortas of group III and eight of group IV were collected from pathological-anatomical laboratories in other parts of the country and they had been stored for several days, at —30 °C, before dissection was performed.

Non-aqueous extraction and separation of the lipids (see § 2.6) yielded five phospholipid fractions for each preparation. Application of the thromboplastin generation test (TGT) showed that the fraction eluted from the silicic acid with chloroform and the $12\frac{1}{2}$ % MC* fraction displayed an inhibiting effect (2—10 % and < 2 %) on thromboplastin formation, the 50 % MC and the 80 % MC fractions behaved indifferently (10—20 %), whereas the 20% MC fraction was positively connected with thromboplastic activity.

The results given by the 20 % MC fraction in the TGT are presented in table 9. In groups I and II, thromboplastic activity was greater than 200 % (clotting time shorter than 7 seconds). In groups III and

* MC = methanol-chloroform.

IV thromboplastic activity was present but it displayed a lower potentiality (66—91 %). The pure media preparations IV^m may be slightly more active (91 %) than the preparations of intima IV^i contaminated with medial tissue (73 %).

Table 9. *Thromboplastic activity of the* 20 % *MC phospholipid fraction of non-diseased tissue (in* %)

No. Preparation	Concentration of phospholipid in Thromboplastin Generation Test				Further Observations
	1000 γ/ml	300 γ/ml	100 γ/ml	30 γ/ml	
I*	> 200	> 200	146	20 — 30	In this case for 30 % read: 20 %. 12 aortas from 0—30 days old subjects.
II	> 200	> 200	172		7 aortas, from 0 days — 10 months old subjects.
III	66	62	20 — 30		13 aortas from 0 days — 10 months old subjects.
IV^i	73	20 — 30	10 — 20		Intimal layer (IV^i) and media (IV^m) from 18 aortas from 0 days to 21 months old subjects.
IV^m	91	20 — 30	10 — 20		

* In this case phospholipids were separated from other lipids by silicic-acid chromatography instead of by dialysis.

DISCUSSION

The investigations, both bibliographic and experimental, presented in this dissertation form part of a larger project for atherosclerosis-thrombosis research carried out by a team primarily engaged in examining various aspects of the relationship between lipids and occlusive vascular disease. The work described here represents the reconnoitring phase in the examination of still another aspect. It comprises an exploration of the presence of thromboplastic activity in aqueous extracts of the arterial wall, diseased and non-diseased, and inquires into the possible lipoid nature of the thromboplastic substance; furthermore the thromboplastic activity of lipid components isolated from the arterial wall, diseased and non-diseased, has been investigated.

Histopathology of the vessel wall has now reached the stage of demonstrating that fibrin is present in even the smallest atherosclerotic lesion. Although a participation of the wall in the early stages of thrombogenesis has often been suggested, little definite evidence on this score has so far been obtained. What little attention has been given to this problem has been mainly directed to the study of homogenates of the arterial wall. We have also followed this line of approach and propose in the following to compare our results with those obtained by others, with respect both to the methods used and to the conclusions reached.

Astrup et al. (1959) and Stevenson et al. (1961) have used recalcification tests comparable with the one-stage system (Quick) employed by us. These authors found no evident differences in thromboplastic activity between 'normal' and atherosclerotically affected tissue. However, Stevenson et al. reported that the thromboplastin generation test (TGT) did reveal a difference, the atherosclerotic material yielding a higher activity. Our results are not strictly comparable with those quoted, for these authors homogenized with a saline solution having the usual pH of distilled water of less than 6, while we used a buffered medium of pH 7.3 which also contained citrate in order to remove

calcium ions and so avoid the formation of intermediate coagulation products from components of the blood present in the tissues. We are in agreement with these authors that recalcification tests lead to variable results, although in this type of research Quick's one-stage system is useful as a rapid screening method to distinguish between indifferent materials and those which display activity.

The TGT, as applied to our preparations, revealed the presence of intrinsic thromboplastic activity both in the intimal layer of aortic sections from patients who underwent an operation because of arterial obstruction of a severe degree (Table 2) and in that of relatively non-atherosclerotic sections from young individuals (Table 7). In one aorta obtained at autopsy the stratum of the arterial wall contiguous to the lumen was not active. Although it would certainly not be justified to draw conclusions from this single observation, it seems in general more desirable to work with fresh material than with autoptic, for we have found that the substances on which thromboplastic activity in the homogenate depends are not stable whatever precautions are taken to preserve it. For this reason all assays of the homogenates of one section were performed within a period of six hours.

The results obtained with homogenates of the media are even more variable than those of the intimal layer, ranging from considerable activity to complete inhibition of clotting, but thromboplastic activity was in all cases less than that of the corresponding intimal layer. Astrup et al. and Stevenson et al. reported similarly that the media was less active than the intima.

The third method of assay, thrombelastography, mimics best what might occur in vivo. In the procedure followed by us the influence of the tissue material on the different phases of blood coagulation is measured in the presence of almost undiluted coagulation inhibitors; the suspension applied is only one tenth of the volume of the blood, whereas in the other two assays equal volumes are mixed. Furthermore in thrombelastography triggering of the very first phase of coagulation determines the clotting time more than does the amount of thromboplastin formed. The fact that the results found in the TGT and in Quick's one-stage system are not always paralleled by those obtained with the thrombelastograph (e.g. Table 7) becomes therefore comprehensible: the assay methods stress different things.

It becomes clear from our investigations as well as from those of others that homogenates, both of atherosclerotic and of non-atherosclerotic aorta may contribute to the formation of throm-

boplastin. Actually it is more remarkable that undiseased intimal tissue should exhibit thromboplastic activity than that atherosclerotic material should do so, for the latter is after all to be expected. In tissue modified by atherosclerosis, vascularization is known to take place, accompanied by haemorrhage and other processes which result in the local deposition of corpuscular elements from the blood. Thrombocytes as well as erythrocytes are known to contain highly thromboplastic compounds which are not in action in physiological circumstances but become effective when the cell membrane is damaged. This factor may well provide an explanation of the variability in thromboplastic acitivity found by us and by others, and further investigations of the thromboplastic potentiality will have to take into account the relative amounts of cellular elements present normally and under pathological circumstances.

Summarizing the practical results of this part of our exploratory work we come to the following conclusions.

(*a*) The method of dissection of layers of the arterial wall with scalpel and forceps is liable to fall short of a true separation along histological lines; this is particularly so in the case of the intima.

(*b*) In the preparation of (aqueous) extracts from arterial tissue the use of a Potter homogenizer proved satisfactory, especially for small amounts (5—500 mg) of tissue. As a homogenization medium we prefer a buffered solution of pH 7.3, approximating a physiological ionic composition and which in addition contains citrate (see § 2.2). Of the authors who examined the thromboplastic activity of homogenates only Astrup mentions the concentration of tissue (200 mg per ml). The proportion of 100 mg tissue per 2 ml homogenization medium tried out by us proved satisfactory; from the tests performed on dilutions of the homogenates it appears that half this concentration would also be suitable.

(*c*) In checking up on the stability of the homogenates we found, as has been mentioned by others, that thromboplastic activity declines rapidly, whatever precautions are taken regarding temperature of storage (e.g. + 2 °C, — 30 °C). It follows therefore that comparability of results suffers if the homogenates are tested at an arbitrary interval after their preparation has been completed. We are in agreement with Astrup that estimations of thromboplastic activity immediately after the preparation of homogenates has been completed yield inconsistent results compared with preparations which have been allowed to settle.

We have endeavoured to gain information concerning the nature of the substance responsible for thromboplastic activity in the homogenates. From two preparations (I and II) the aqueous extract was treated according to Folch et al., by which method lipids are separated from non-lipids. Neither of the moieties derived from the two preparations showed any trace of thromboplastic activity. Extraction and fractionation of the tissue residue left behind after the homogenate is poured off, yielded negligible activity.

Howell (1912) and subsequently others (e.g. Gratia and Levene 1922) suggested that the substance responsible for thromboplastic activity of aqueous tissue extracts consisted of an active phospholipid (cephalin), bound to a protein. In 1942 Chargaff et al. isolated a lipoprotein with thromboplastic activity from saline extracts of lung tissue. The loss of thromboplastic activity, which we found when the homogenates were fractionated by the Folch procedure, may well be explained by assuming that the substance responsible for activity is a lipoprotein, which is denatured by the solvents used. In extracting and fractionating the phospholipids from homogenates we failed to isolate a phospholipid which showed thromboplastic activity. Thus we were unable to confirm that the essential substance responsible for activity of the homogenate is a phospholipid. It appeared from the fractionation of the tissue residue that few, if any, active phospholipids were left in the tissue after homogenization was completed.

We have concentrated on examining the phospholipid fractions from the arterial wall in respect to thromboplastic activity after preliminary investigations had ruled out the possibility that any of the other lipid fractions isolated from aortas had any bearing on the coagulation mechanism, as projected in vitro.

From each of the atherosclerotic and non-atherosclerotic preparations five phospholipid fractions were separated by column chromatography on silicic acid. Coagulation assays showed that the first of these fractions, as well as the two last ones consistently behaved indifferently in the tests. The third (20 % MC) fraction proved to be active and the second (12½ % MC) fraction inhibitory in nine atherosclerotic preparations, in one thrombus, and in the combined preparation of atherosclerotic lesions; all fractions derived from three atherosclerotic preparations and from one thrombus behaved indifferently. Of four groups of non-atherosclerotic, very young aortas (split in one case into intimal layer and media preparations) the 20 % MC

fraction again displayed thromboplastic activity coupled with an inhibitory effect on thromboplastin formation in the $12\frac{1}{2}$ % MC fraction.

Both the $12\frac{1}{2}$ % and the 20 % MC fractions are mixtures of extreme complexity. The contradictory statements made by many authors (see § 1.2) as to the identity of phospholipids active in thromboplastin formation in vitro, bear evidence of the confusion existing in this field of research.

It is tempting to speculate that differences in the composition of the 20 % MC fractions are responsible for the variability of the results obtained by applying the fractions to the thromboplastin generation test. It is hardly conceivable, however, that the total absence of thromboplastic activity in four out of eighteen fractions could be explained in this way. Considering the discrepancies found by us and by others (e.g. Biggs and Bidwell), we incline to the views of those (e.g. Rouser and Schloredt; Wallach et al.) who attribute the behaviour of phospholipids in the coagulation mechanism to physicochemical properties rather than to their specific chemical aspect. It would be imprudent to reject the possibility that essential structural properties of phospholipids, pertaining for instance to the formation of micelles, may run the risk of being affected by the fractionation methods applied, although we have no evidence that this in fact occurred.

Although the percentages of thromboplastin formed in assays of phospholipid fractions were variable, the collected data show conclusively that phospholipids associated with thromboplastic activity are present in the atherosclerotic aorta of patients with severe symptoms of arterial obstruction as well as in the non-atherosclerotic aorta of the young child.

Summing up the practical conclusions of the section concerning the thromboplastic activity of lipid components isolated from the arterial wall:

(*a*) We do not see our way clear to comparing the methodology of the separation and fractionation as performed on the phospholipids in the present investigation with similar work reported by others. Not only is our source of phospholipid the human arterial wall, as opposed to the various tissues selected by other authors, but in addition the methods of separation applied vary from paper chromatography, with subsequent elution of the spots, via countercurrent distribution, to column chromatography with other eluting systems

than those used by us. Marcus and Spaet have eluted on silicic acid with methanol-chloroform 20 % which yielded a group of phospholipids, part of which displayed marked thromboplastic activity as shown by the TGT. This is in agreement with our finding of activity in the third fraction, eluted with methanol-chloroform 20 %. Turner and Silver and Tocantins have stated repeatedly that they had isolated an 'antithromboplastin', an inhibitor of thromboplastin formation, from brain tissue. We have found that the fraction eluted with 12½ % methanol-chloroform consistently displays an inhibitory effect on thromboplastin formation.

(*b*) We have chosen to suspend 1 mg of phospholipid in 1 ml of Veronal buffer (see § 2.2) for testing on thromboplastic activity; this proportion proved satisfactory but from the tests performed on the 30 % dilution of suspensions it may be concluded that in further investigations a lower concentration, e.g. 0.5 mg per ml, would also be suitable.

(*c*) Although the suspensions of phospholipid proved to be not quite as unstable as the homogenates, we found that they could not be stored for long periods, even at —30 °C. Thromboplastic activity also disappears from phospholipid fractions stored, at —30 °C, in methanol-chloroform instead of in aqueous suspension. It has been reported previously (e.g. Ferguson 1960) that fractionated phospholipids are unstable in this respect, unlike 'crude cephalin', which may remain stable for years, as we have also found.

The assay methods used for the estimation of thromboplastic activity were of necessity borrowed from clinical haematology and tried for their usefulness in this type of research. In order to avoid confusion we stress again that the events in a clotting assay system cannot be regarded as even a model of what actually happens in vivo. 'The intention with a clotting system is to characterize an unknown component in the system, the test substance, either as to coagulability, one specific activity or a combination of activities' (Waaler 1959). Although with the help of these tests we have been able to discover something about the thromboplastic activity of the substances in the arterial wall, we as yet know nothing of the specific effect these substances may have on either the extrinsic coagulation system or the intrinsic coagulation system. In further explorations it would be necessary, in order to obtain information concerning the specific effect of the tissue extracts, to use as coagulation substrate not only normal plasma and blood but also plasma and blood lacking in

factor VII or in factor XII. In the first case the extrinsic pathway would be blocked in the clotting system, in the second case the intrinsic pathway. Both the TGT and thrombelastography seem satisfactory as test methods. Whereas the TGT registers mainly the amount of thromboplastin formed, thrombelastography stresses the triggering effect on the generation of thromboplastin.
For completeness sake it should be mentioned here that in the course of the present investigation a few data (insufficient for presentation here) were collected concerning the activation of plasma, deficient in factor VII, which gave the impression that the homogenates do have some influence on factor VII, i.e. on the extrinsic coagulation system, whereas the suspensions of (active) phospholipids have not.

While we can expect that progress will be made along the lines proposed for the further differentiation of thromboplastic activity of the arterial wall, there is no doubt that methods currently being developed for the fractionation and characterization of phospholipids will be useful and indeed necessary for further elucidation of the problem.

SUMMARY

The bibliographic review (Chapter I) reveals that morphological studies have now demonstrated the presence of fibrin besides lipoid elements in even the first atherosclerotic lesions. Furthermore it is shown that few specific data exist concerning the relationship between atherosclerosis and thrombosis which might explain the rôle played by these substances in occlusive vascular disease. For this reason it seemed useful to examine the thromboplastic properties of atherosclerotic and non-atherosclerotic tissue; the thromboplastin generation test, the prothrombin-time determination and thrombelastography were used as coagulation assays.

Of the *homogenates* of three atherosclerotic and two relatively non-atherosclerotic aortic preparations obtained at operation, all displayed thromboplastic activity, of varying degree, in the intimal layer and in the media; another non-diseased aorta, which was obtained at

autopsy, showed moderate activity in the homogenate of the intimal layer, but a strong inhibitory effect in the homogenate of the media. No marked difference between thromboplastic activity of atherosclerotic and relatively non-atherosclerotic tissue was found in these instances.
We were unable to demonstrate thromboplastic activity of the (phospho)lipids isolated from two of these homogenates.

The *phospholipids* were extracted from twelve atherosclerotic sections of the bifurcation of the aorta, from two thrombi detached from the wall of two of these sections, from a combined preparation of atherosclerotic lesions, and from four groups of non-atherosclerotic, very young aortas, and were fractionated by silicic-acid chromatography. In the fraction eluted with 20 % methanol-chloroform thromboplastic activity was found in all preparations, except in three of the atherosclerotic sections and one thrombus. Possible reasons for the absence of activity in these four preparations are discussed. The phospholipids from the non-atherosclerotic young aortas possessed considerable thromboplastic activity; one group of these aortas was separated into intimal-layer and media preparations, both of which showed thromboplastic activity of approximately the same degree.
We have come to the conclusion that the thromboplastic potentiality of the aortic wall of the young child is at least equal to that of the aortic wall of patients with arterial obstruction of a severe degree, at least as far as this activity can be attributed to the presence of thromboplastic phospholipids.

The practical implications of this reconnoitring work are discussed and suggestions are made concerning further exploration of this field of research.

SAMENVATTING

Hoewel het voorkomen van fibrine naast dat van lipoide elementen door morphologische onderzoekingen is aangetoond, bestaan er slechts weinig gegevens omtrent het verband tussen atherosclerose en thrombose van de arterie, waardoor de aanwezigheid van deze beide substanties verklaard zou kunnen worden, zoals uit het literatuuroverzicht blijkt (Hoofdstuk I). Het leek ons daarom nuttig te

trachten iets meer te weten te komen betreffende de thromboplastische eigenschappen van de vaatwand bij oblitererende vaatziekten. Als stollingstests werd hierbij gebruik gemaakt van de thromboplastin generation test, van de prothrombine-tijd bepaling en van thrombelastographie.

De *homogenaten* van drie atherosclerotische en twee niet-atherosclerotische operatiepreparaten van de aorta vertoonden alle thromboplastische activiteit, in uiteenlopende mate, zowel van de intimale laag als van de media. Eén niet-atherosclerotische aorta, die afkomstig was van een obductie, vertoonde een matige activiteit van de intimale laag, een sterk remmend effect daarentegen van de media.
Er was geen duidelijk verschil waarneembaar in thromboplastische activiteit tussen de homogenaten van atherosclerotisch en van niet-atherosclerotisch weefsel.
In de (phospho)lipidfracties, geïsoleerd uit twee van deze homogenaten, konden wij geen thromboplastische activiteit aantonen.

De *phospholipiden*, geëxtraheerd en vervolgens met behulp van kolomchromatographie gefractioneerd, van twaalf atherosclerotische gedeelten van de bifurcatio aortae, van twee thrombi, van een gecombineerd preparaat van atherosclerotische laesies en tevens van vier groepen niet-atherosclerotische zeer jonge aorta's werden onderzocht op thromboplastische eigenschappen. Van al deze preparaten was de fractie geëlueerd met 20 % methanol-chloroform thromboplastisch actief, uitgezonderd van drie van de atherosclerotische aortagedeelten en van één thrombus. Een mogelijke verklaring voor het ontbreken van activiteit in deze vier preparaten wordt besproken.
De phospholipiden van niet-atherosclerotische jonge aorta's bleken eveneens thromboplastische activiteit te bezitten; één groep van deze aorta's werd onderscheiden in intimale en mediale laag, die nagenoeg evenveel thromboplastische activiteit vertoonden.
Uit dit gedeelte van ons onderzoek wordt duidelijk, dat de aortawand van de patiënt met een oblitererend vaatlijden niet de aortawand van het zeer jonge kind overtreft in thromboplastische activiteit, dat wil zeggen voor zover deze afhankelijk is van het in de arteriewand aanwezige phospholipid.

De practische conclusies van dit oriënterend onderzoek worden besproken en mogelijke richtlijnen aangegeven voor verdere research op dit terrein.

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VII

Bij een onderzoek naar de chemische samenstelling der lipiden in de verschillende arteriegebieden bleek, dat in het ene arteriegebied een andere groep lipiden op de voorgrond treedt dan in het andere.

(Böttcher J. C. F., Boelsma-van Houte E., ter Haar Romeny-Wachter C. Ch., Woodford F.P., van Gent C.M., *Lancet* 1960 II - 1162)

VIII

Het doorgankelijk maken van de arteria profunda femoris is van essentieel belang voor het behoud van een ischaemische onderste extremiteit.

IX

Voor de diagnostiek van de hypertensie berustend op een stenose van de arteria renalis is de aortographie essentieel; de z.g. Howard-test kan slechts aanvullende informatie geven.

(Annotation *Lancet* 1961 I - 548)

X

De moderne immuno-fluorescentie van het primair atypische pneumonie 'virus' (het z.g. Eaton-agent) maakt een snelle diagnose op het sputum mogelijk; zowel hierdoor als door het titerbeloop der antistoffen kan een beter inzicht worden verkregen in de klinische aspecten van deze respiratoire infectie.

XI

Een reincultuur van angiotheelcellen te kweken blijkt tot op heden niet mogelijk.

XII

Het gebed, waaraan inherent is een ervaren van de betrekkelijkheid van eigen zijn en doen, biedt bij uitstek de mogelijkheid tot 'ontspanning'.